Detection of Rickettsiae in Arthropod Vectors by DNA Amplification Using the Polymerase Chain Reaction[a]

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INTRODUCTION

Rickettsial infections are prevalent throughout the world and cause serious diseases in humans. Most of the pathogenic rickettsiae are transmitted to humans by a diverse group of hematophagous arthropods.[1] Arthropod vectors such as ticks, fleas, and blood-sucking mites are involved in the maintenance of the pathogenic rickettsiae and their transmission. Therefore, the detection of pathogenic rickettsiae in the vector arthropods is of major importance for epidemiological studies and control strategies. A number of antigen-detection techniques have been developed, including direct (DFA) or indirect immunofluorescence (IFA) tests[2,3] and enzyme-linked immunosorbent assays (ELISA) using polyclonal or monoclonal antibodies.[4] Other techniques, such as recovery of the agents from vectors by culture in embryonated eggs or tissue culture cells or by experimental infections in laboratory animals have been used extensively.[2,4] DFA technique is currently the method most commonly employed. However, DFA as well as IFA require examination of the gut from live arthropods. Dissection and preparation of specimens from live arthropods can be hazardous and require extensive laboratory support and antisera to well-defined antigens. These techniques are also time consuming and expensive. In addition, the recovery of the pathogenic organisms from infected vectors requires either live or properly frozen specimens and their delivery to the laboratory facilities.

The application of the polymerase chain reaction (PCR), which uses specific oligonucleotide primers and *Taq* DNA polymerase to synthesize copious quantities of DNA from a single template,[5] proved to be a valuable new approach to the detection and identification of pathogenic rickettsiae within infected vectors. Using the published *Rickettsia rickettsii* 17-kDa-antigen gene sequence,[6,7] we synthesized two oligonucleotides 20 bases in length that are complementary to two

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regions separated by 434 base pairs (bp) on opposing strands of the 17-kDa-antigen gene.[8] Since the 17-kDa antigen is common to members of both the spotted fever and the typhus group rickettsiae,[6,7] we expected this one primer pair to detect rickettsiae in either group in their respective arthropod hosts. We previously demonstrated the utility of this primer pair for detecting *R. typhi* in fleas[8] and *R. prowazekii* in clinical specimens.[9] The utility of the PCR was further enhanced by elimination of the need for live arthropods and the isolation of purified DNA from them, and by development of a sensitive dot hybridization assay for the PCR product.[8] Here we further demonstrate the value of the flea PCR assay and demonstrate that it can be used without modification to detect *R. rickettsii* in infected ticks.

MATERIALS AND METHODS

Rickettsial Strain

Rickettsia typhi strain AZ 332 (Ethiopian)[10] was used to infect fleas. Infected *Xenopsylla cheopis* fleas were obtained after feeding them on rickettsemic laboratory rats as described previously.[4,8,10] Flea samples were collected at day 0 and every other day thereafter (days post-infection: dpi) and stored in tubes containing 0.5 ml brain heart infusion broth (BHI) at −70°C. *Rickettsia rickettsii*–infected *Dermacentor variabilis* was kindly provided by Dr. Tom G. Schwan from Rocky Mountain Laboratories, Hamilton, Montana.

Diagnosis by PCR

Selection of Sequences for Primers and Probes

A pair of 20-residue oligonucleotide primers (primer #1: 5′-GCTCT-TGCAACTTCTATGTT-3′; and primer #2: 5′-CATTGTTCGTCAGGTTGGCG-3′) were synthesized according to the published DNA sequence of the gene encoding the 17-kDa protein antigen from *R. rickettsii*[6] (FIG. 1). Each of the two 20-base oligomer primers was complementary to a region of DNA where *R. rickettsii*, *R. conorii*, *R. prowazekii*, and *R. typhi* have very similar nucleotide sequences (base 10 of primer #1 is G for *R. typhi* and *R. prowazekii*; base 6, 9, and 18 of primer #2 are C, A, and A, respectively, for *R. prowazekii*).[7] Therefore, this pair of primers was capable of amplifying *R. rickettsii* and *R. typhi* DNA. The length of the rickettsial genome targeted for amplification was predicted to be 434 bp.[9] The specificity of these primers was tested in PCR using purified DNA from various bacteria. A single band with the length predicted for amplified DNA was obtained with *R. rickettsii*, *R. prowazekii*, *R. typhi*, and *R. canada* (McKeil) but not with *Ehrlichia risticii* (ATCC VR-986), *Escherichia coli* (K12), *Wolbachia persica* (ATCC-VR 331), *Rochalimaea quintana* (Fuller), and *R. tsutsugamushi* (Karp, Kato, and Gilliam strains).[8,9]

Preparation of Samples for PCR Analysis

Individual ticks and fleas (infected and uninfected) were triturated in 100 μl of BHI and boiled for 10 min. The PCR was carried out using 10 μl of the boiled suspension. Serial tenfold dilutions of a yolk sac seed AZ 332 strain of *R. typhi*

were used to establish the sensitivity of the PCR. Control DNA was prepared from *R. rickettsii*–infected *D. variabilis* ticks and *R. typhi*–infected fleas by proteinase K/SDS digestion followed by repeated phenol-chloroform extractions and ethanol precipitation. The presence of rickettsiae in individual ticks and fleas was determined by DFA using fluoroscein isothyocyanate–labeled anti–*R. rickettsii* and anti–*R. typhi* (convalescent) guinea pig sera.

Amplification

PCR was run as described previously on a Thermal Cycler (Perkin-Elmer Cetus, Norwalk, CT).[8,9] Each 100-μl sample was amplified for 35 repeated cycles of denaturation at 94°C for 30 s, annealing at 57°C for 2 min, and sequence extension at 70°C for 2 min in the presence of 2 units of *Taq* polymerase (Perkin-Elmer Cetus, Norwalk, CT) and each of the four deoxynucleotide triphosphates in the reaction mixture (100 μl total).

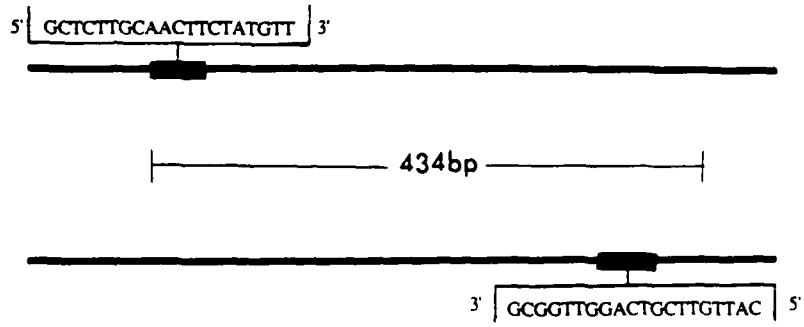

FIGURE 1. Sequences of primers #1 and #2 and their relative positions on the targeted 17-kDa-antigen gene of *R. rickettsii* DNA. The 20-base oligonucleotide primers permit amplification of DNA extending from nucleotide 31 through nucleotide 534 of the reading frame of the 17-kDa-antigen gene.

Detection and Identification of the 17-kDa Sequence in Reaction Products

The target DNA sequence amplified by PCR was identified by visualization of a 434-bp band on agarose gels after electrophoresis.[8,9] In the agarose gel method, 14 μl of the PCR product was subjected to electrophoresis in 1% gels (SeaKem: FMC Bioproducts, Rockland, ME), and the gels were stained with ethidium bromide and examined for bands of appropriate size using UV transillumination.

RESULTS AND DISCUSSION

Our earlier studies revealed that extraction of purified DNA from either fleas or rickettsial seeds was not necessary in the PCR assay.[8] For example, DNA extracted from 1×10^7 plaque-forming units (PFU) of rickettsiae produced a more faint PCR band in agarose gels (data not shown) compared to the band

produced by the same quantity of rickettsiae which had been boiled for 10 min prior to PCR assay and used without further processing. Consequently, we have used this simplified sample treatment throughout this study. Furthermore, as we have reported previously, agarose gel electrophoresis of the PCR products revealed the predicted band of 434 bp for samples containing 10^5 through 10^2 rickettsial PFU/ml,[8,9] but no band was seen for greater dilutions. Even greater sensitivity was obtained when dot hybridization was used to assay amplification products, since even one rickettsial PFU/ml of the reaction mixture could be detected.[8] Comparable results were obtained in the present study (FIG. 2). *R. typhi*–infected fleas from different batches with known rickettsial PFU/flea or ELISA titers were used in the PCR reaction. The infected flea samples, BHI seeded with 10^6 *R. typhi* PFU (data not shown), and 9-, 10-, and 14-day infected fleas revealed a band of the expected size (434 bp) (FIG. 2, lanes 4, 5, 7, 8). The reaction products from uninfected individual flea specimens did not contain a detectable 434-bp band in agarose gels (FIG. 2, lanes 3 and 6), thus excluding that amplification of the PCR product was from the endogenous microflora and endosymbionts of the flea.

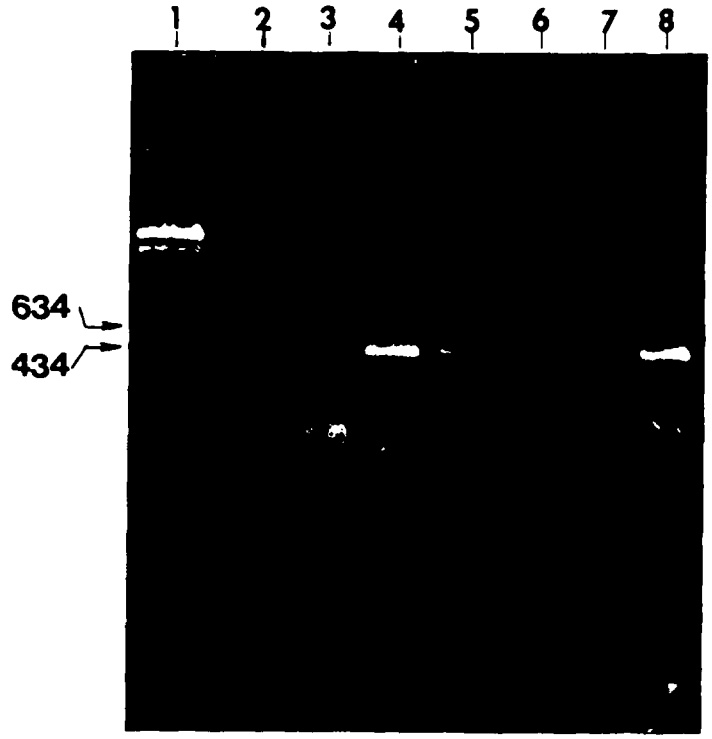

FIGURE 2. Agarose gel electrophoresis of PCR-amplified products from infected and uninfected fleas. **(Lanes 1 and 2)** Molecular size markers and blank lane. **(lanes 3 and 6)** uninfected fleas, and **(lanes 4, 5, 7, and 8)** infected fleas: 10 dpi *X. cheopi* , 14 dpi *X. cheopis*, 10 dpi *Ctenocephalides felis*, and 9 dpi *X. cheopis* fleas, respectively. Fleas were surface sterilized by repeated washing in 70% ethyl alcohol and BHI and then homogenized in 100 μl of BHI. This preparation was then boiled for 10 min and used in the PCR procedure.

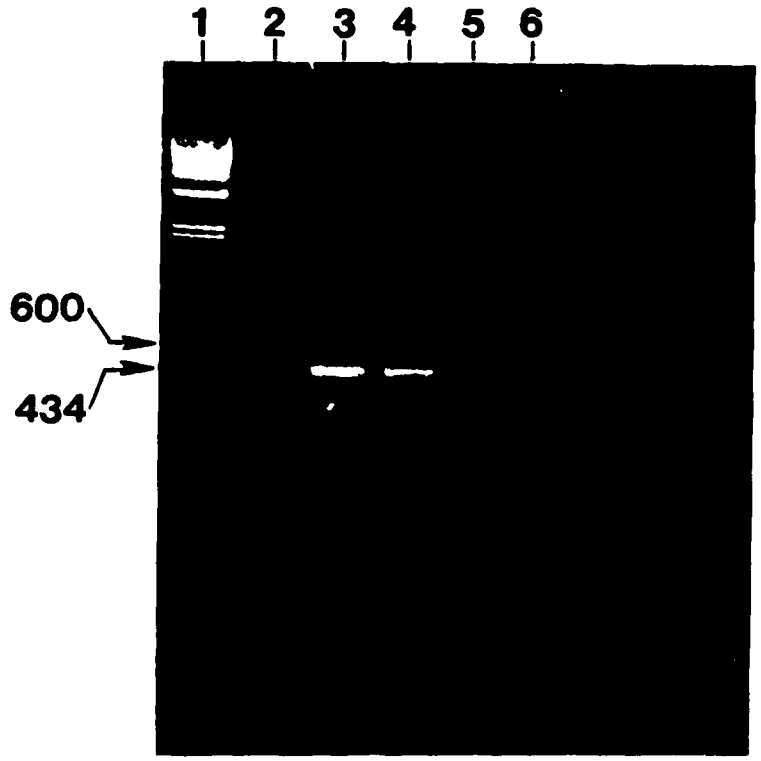

FIGURE 3. Agarose gel analysis of PCR products of *R. rickettsii*–infected and uninfected *Dermacentor variabilis* ticks and *R. rickettsii*–infected and uninfected Vero cells. **(Lane 1)** Molecular size markers, **(lane 2)** DNA extracted from 4 infected ticks, **(lane 3)** Vero cells inoculated (7 days post-inoculation) with 0.2 ml of infected tick homogenate, **(lane 4)** individual infected tick, and **(lanes 5 and 6)** uninfected Vero cells and uninfected ticks, respectively.

Similar studies were performed using *R. rickettsii*–infected ticks. The results are shown in FIGURE 3. Agarose gel analysis of the PCR products revealed the expected 434-bp band obtained with purified DNA from infected ticks (lane 2), boiled extracts of Vero cells inoculated with the infected tick homogenate (lane 3), and boiled infected tick homogenate (lane 4). In contrast, neither the PCR-amplified samples of uninfected ticks nor those of uninfected Vero cells contained a band of the expected size (lanes 5, 6). All the ticks which were positive by PCR ($n = 4$) were also positive by DFA.

The proven sensitivity and specificity of the PCR procedure makes it a very useful tool for the detection of *R. rickettsii* and *R. typhi* infection in vector ticks and fleas. It provides several advantages over other well-established, sensitive, and specific assays, namely ELISA and DFA. Unlike ELISA, DFA and plaque assays, this technique does not require fresh or properly frozen specimens. The assay may possibly even be suitable for detecting rickettsiae in the dry or preserved specimens often obtained in field surveys. Moreover, the PCR assay can

reduce the potential dangers involved in the maintenance and transportation of the infected vectors from the field to the laboratory. For example, dead fleas and ticks could be collected for PCR assay following insecticide application.

The present conserved 17-kDa-antigen PCR primer pair should be suitable for most clinical applications, since it is unnecessary to distinguish typhus and spotted fever rickettsiae for appropriate medical care of patients. However, an understanding of the epidemiology and natural history of rickettsiae will require more specific PCR assays that can distinguish not only typhus and spotted fever rickettsiae, but also different pathogenic and non-pathogenic rickettsiae found in ticks.[1,2] Additional PCR primers, including those derived from genes less conserved than the 17-kDa-antigen gene, or supplementation of the PCR assay by analysis of the PCR product with specific probes or restriction enzyme digestion, may be necessary to identify individual species of rickettsiae. Nonetheless, these additional approaches only represent refinements of the present PCR technology, which works quite effectively in detecting the often scant quantities of rickettsiae present in their arthropod vectors.

CONCLUSION

Polymerase chain reaction (PCR) amplification of DNA was used to detect *Rickettsia rickettsii* and *R. typhi* in experimentally infected adult *Dermacentor variabilis* ticks and *Xenopsylla cheopis* fleas, respectively. A primer pair derived from the 17-kDa-antigen gene sequence of typhus and spotted fever group rickettsiae was used to amplify a 434–base pair (bp) fragment of the genome of the rickettsiae. The specific PCR-amplified product in extracts of individual infected fleas or ticks was detected readily on ethidium bromide–stained agarose gels. The amplified 434-bp sequence was not detected in uninfected controls. The PCR procedure provides a rapid, sensitive, and highly specific assay for detection of rickettsial infection in arthropod vectors.

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